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A Novel Stool PCR Test for *Helicobacter pylori* May Predict Clarithromycin Resistance and Eradication of Infection at a High Rate

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ABSTRACT Clarithromycin-based regimens are commonly used as a first-line therapy for *Helicobacter pylori*-positive patients; however, resistance to clarithromycin has led to treatment failures. The aim of this study was to evaluate the feasibility of using stool samples to detect the presence of *H. pylori* DNA while concurrently detecting mutations associated with resistance to clarithromycin. For this purpose, total DNA was extracted from 294 raw stool specimens from *H. pylori*-positive and -negative patients. TaqMan real-time PCR amplification was used to detect the presence of *H. pylori* as well as to predict the phenotype of the organism and the related outcome for patients treated with clarithromycin. Clarithromycin resistance was determined upon analysis of the PCR result. Patients were also tested by a urea breath test and were subjected to esophagogastroduodenoscopy, followed by histology, culture, and a rapid urease test, in order to obtain a consensus patient infection status. Of 294 total stool samples, 227 were deemed true positive. The sensitivity of *H. pylori* detection by PCR was 93.8%. Of 213 true-positive samples that were sequenced, 36.2% showed point mutations associated with clarithromycin resistance (A2142C, A2142G, A2143G). The final correlation of the mutant genotypes as determined by sequencing with the eradication of infection was 86%. We found that *Helicobacter pylori* DNA can be detected in human stool specimens with high sensitivity and can therefore be used to determine the presence of the bacterium without obtaining a biopsy sample. Moreover, genotypic resistance to clarithromycin can be predicted without obtaining a biopsy sample, facilitating the choice of the right therapeutic approach.

KEYWORDS *Helicobacter pylori*, stool PCR test, clarithromycin resistance, antibiotic resistance, clarithromycin, polymerases, stool

Helicobacter pylori infection is a major cause of gastric ulcer disease and gastritis in humans and is a risk factor for the development of gastric cancer. Current guidelines recommend that all patients with documented *H. pylori* infection should be treated with appropriate antibacterial therapy (1–4).

Clarithromycin (CLA)-based triple therapy, consisting of a proton pump inhibitor (PPI) and amoxicillin or metronidazole, is commonly used as a first-line treatment; however, *H. pylori* resistance to clarithromycin has gradually increased worldwide (5, 6). Soon after the introduction of clarithromycin-based therapies, treatment failures associated with resistance to clarithromycin were reported (5, 6). Genetic analysis of resistant strains isolated from patients who failed primary clarithromycin-based treatment identified mutations in the 23S rRNA as the predominant cause of resistance (7, 8). In particular, point mutations at positions 2143 (A to G) and 2142 (A to G or C) are responsible for >90% of clarithromycin resistance cases (9). Moreover, the presence of

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TABLE 1 Diagnostic outcome of PCR versus the gold standard and UBT for *H. pylori* detection

H. pylori result by PCR	No. of samples with the following result for *H. pylori* detection by the gold standard/UBT:		
	Positive	Negative	No EGD
Positive	227	11	1
Negative	15	37	1
Invalid	2	0	0

the A2143G mutation is strongly associated with eradication therapy failure (10, 11). A lower prevalence of the A2142G/C mutation has been reported (11), but these mutations are also commonly found in patients for whom clarithromycin-based treatment failed to eradicate the infection. A recent global consensus report on *H. pylori* gastritis recommends assessing the outcome of eradication therapy, preferably using a noninvasive test such as the stool antigen test (SAT) or urea breath test (UBT) (2, 4). In the case of eradication failure, there is a choice between several alternative treatments (5). Testing of *H. pylori* for antibiotic sensitivity has been complicated due to the necessity of performing culture and an antimicrobial susceptibility test, which require additional biopsies and specific laboratory competence.

The approach of using a stool specimen in a molecular test for clarithromycin resistance has been proposed (12). To date, data on the use of such an approach in clinical research are very limited. It appears that while the specificity of the molecular test has consistently been very high (>95%), the achievement of high sensitivity remains very challenging (13).

The aim of this study was to evaluate the feasibility of using stool samples to detect *H. pylori* DNA while concurrently detecting mutations associated with resistance to clarithromycin.

RESULTS

Stool specimens were collected from January 2015 to January 2016 and were tested by PCR within a month of collection. The optimal amount of stool, producing the best sensitivity of detection while presenting minimal inhibition to PCR, was determined to be approximately 200 mg (180 to 220 mg, the same as the amount in the manufacturer's instructions) from preliminary experiments (data not shown). In signal analysis of the genotype by PCR, baseline detection of *H. pylori* was represented by the green probe, and a prediction of the genotype was made based on analysis of the red probe (see "Analysis of PCR" below). Genotypic predictions by melt peak temperature were categorized based on the temperature at which the fluorescent signal peak was observed. Predictions were verified by the results of bidirectional sequencing and were compared to eradication outcomes when patients were treated with clarithromycin.

Of 294 total stool samples from *H. pylori* patients, 227 were positive both by the composite reference method (CRM) and by the novel PCR test. Two samples were invalid by PCR. The diagnostic outcome for *H. pylori* detection by PCR is shown in Table 1. PCR resulted in approximately 93.8% (confidence interval [CI], 90 to 96.5%) (227/242) sensitivity in comparison with the CRM (excluding invalid results). Because the current study targeted a predominantly positive patient population in order to establish the clinical sensitivity of the stool PCR method, we did not draw conclusions regarding clinical specificity.

The genotypes for all samples positive by PCR that produced definitive sequencing results (including samples negative by the CRM) are shown in Table 2. Twenty-six samples could not be included in the genotypic analysis due to unevaluable results from PCR and/or sequencing. Seven samples were not sequenced or resulted in a failed sequence. Four sequences were unreadable at the nucleotide sites of interest. Nine samples were deemed indeterminate by sequencing or contained mixed alleles. Four samples were discrepant by melting curve analysis and differential signal analysis using

TABLE 2 Genotype results for *H. pylori* PCR-positive stool samples[a]

No. of samples	Genotype[b]	PCR prediction
133	AAA	Susceptible
54	AGA	Resistant
19	GAA	Resistant
3	CAA	Resistant
3	AAA	Resistant
1	AGA	Susceptible

[a]Samples with incomplete, undetermined, or unreadable traces have been excluded. False-positive results by PCR are included.
[b]Positions 2142 to 2144.

PCR determination. Two had indeterminate genotypes by PCR. The CLA susceptibility prediction from PCR was verified by sequencing that established the identity of nucleotides at positions 2142 and 2143 of the 23S rRNA gene. Of the 213 remaining positive samples with evaluable sequencing and PCR results, 77 (36.1%) contained mutations and 136 (63.8%) contained the wild-type sequence. Among the mutations, 3 samples contained the A2142C sequence, 19 samples contained the A2142G sequence, and 55 samples contained the A2143G sequence. One sample that was predicted by PCR to be wild type contained a mutation at the A2143G position. PCR correctly identified 133 of 136 samples (97.8%) containing wild-type (CLA-sensitive) sequence and 76 of 77 (98.7%) samples containing mutations (Table 3).

The correlation between genotype prediction by PCR and eradication of infection for patients treated with clarithromycin was 83.5% [(93 + 8)/121]. An additional 3 patients had wild-type *H. pylori* by sequencing and eradication of infection, for a final genotypic correlation of 86% [(96 + 8)/121)].

The potential cross-reactivity of the PCR toward different *Campylobacter* species commonly found in nondiarrheal stool was also evaluated. At a concentration of 10^6 genomic copies in the reaction mixture, analytical specificity was confirmed with the various *Campylobacter* species (data not shown).

DISCUSSION

These data demonstrate that *Helicobacter pylori* DNA can be detected in human stool specimens with high sensitivity and therefore can be used to determine the presence of *H. pylori* with a performance similar to that of other direct assays, such as the SAT or UBT (14). More importantly, genotypic resistance to clarithromycin can be detected, enabling the right therapeutic approach to be chosen without a biopsy sample. This finding confirms previous reports describing the use of PCR detection and assessment of clarithromycin sensitivity using stool specimens (15).

The design for this PCR test allows for multiple ways to analyze the data. Melting of the red probe targets the mutation sites associated with resistance. The proprietary nature of the TaqMan probes improves melt resolution, enabling a CLA susceptibility prediction (16). The prediction based on a melting curve can be determined immedi-

TABLE 3 Proportions of various groups of patients with *H. pylori*-positive stool samples

Group characteristic[a]	No. of samples	Proportion (%)
Evaluable sequencing and eradication results	121	
H. pylori mutations (A2142C, A2142G, A2143G) determined by sequencing	21	17.4
Wild-type *H. pylori* determined by sequencing	100	82.6
H. pylori with mutations, not eradicated[b]	8	38.0
Wild-type *H. pylori* determined by PCR, eradicated[c]	93	93.0

[a]All groups consisted of *H. pylori*-positive, CLA-treated patients.
[b]PCR and sequencing found equal numbers of samples in this group. The proportion given is the percentage of all samples with *H. pylori* mutations.
[c]An additional 3 patients had wild-type *H. pylori* by sequencing and eradication of infection, for a total of 96. The proportion given is the percentage of all wild-type *H. pylori* samples.

ately upon analysis using Rotor-Gene software. Alternatively, resistance can be predicted using fluorescent signal analysis differentiation as described in Materials and Methods (17). Discrepancies between sequencing results and PCR prediction of resistance may be due to nonspecific traces and technician interpretation. This study also shows a significant correlation between the detection of 23S rRNA mutations conferring resistance to clarithromycin and the final eradication status of the patient. Taking into account the expected bias of this study due to the fact that the majority of the patients predicted to be resistant were not treated with clarithromycin, the drop in the response rate from 93% (for patients with predicted CLA-sensitive organisms) to 62% (13 of 21 patients with resistant mutations) suggests the beneficial impact of the PCR test in the selection of the appropriate therapy. Further clinical studies with a more-balanced patient population with investigators blinded to the outcome of the resistance determination would define the true clinical value of the stool PCR test.

The high analytical specificity and clinical sensitivity of this molecular *H. pylori* resistance assay provide a strong likelihood of accurately predicting eradication of infection by CLA therapy. This assay can be used as a stand-alone screening tool, since it can detect the presence of *H. pylori* as well as resistance, but is most cost-efficient once negative patients are eliminated by a stool antigen test or UBT. Both approaches quickly eliminate negative patients from further screening and predict susceptibility to clarithromycin using noninvasive sample types (14–19).

This PCR test using stool specimens is capable of sensitive detection of *H. pylori* DNA in stool while simultaneously detecting mutations causing clarithromycin resistance. There is a significant correlation between the presence of such mutations and the outcome of clarithromycin-based eradication treatment. The results of this study suggest that stool can be used to determine resistance with a high correlation to eradication status. This stool/PCR test has the potential to reduce health care costs with an updated algorithm. A biopsy sample requires the patient to return to the health care provider for an invasive procedure if treatment is unsuccessful (3). The PCR test option with the noninvasive sample can significantly reduce the need for biopsy samples, currently used to determine resistance in patients who do not need to undergo upper endoscopy according to clinical guidelines (4).

MATERIALS AND METHODS

Samples and extraction method. Stool samples were acquired from a population of patients tested for *H. pylori* infection in Bologna, Italy. Samples were frozen and were kept at −20°C prior to testing. Patients were diagnosed as *H. pylori* positive or negative according to concordant results of the composite reference method (CRM): histology, a rapid urease test (RUT), and culture as the gold standard (14, 18). Total DNA was extracted from 294 raw stool samples using the QIAamp Fast Stool kit (QIAgen), where the workflow matched the protocol for human DNA isolation (instead of the pathogen protocol) as provided by the manufacturer's instructions. A quantitative PCR (qPCR) exogenous control (catalog no. BIO-11025; Bioline) made up of a known concentration of bacterial DNA was spiked into the lysis buffer just prior to extraction. The exogenous control was applicable for high-annealing-temperature PCR and was added during the lysis step of DNA extraction to monitor extraction efficiency and the presence of inhibitors. Extracted DNA was stored at −20°C prior to qPCR testing.

Molecular detection. TaqMan real-time PCR amplification was used to detect the presence of *H. pylori* as well as to predict the phenotype of the organism and the related outcome for patients treated with clarithromycin. Extracts were amplified on the Qiagen Rotor-Gene Q instrument. The PCR master mix comprised an *H. pylori*/resistance primer set (catalog no. ASR100; Meridian Bioscience), an *H. pylori* probe (catalog no. ASR101; Meridian Bioscience), and an *H. pylori* clarithromycin resistance (HPCR) probe (catalog no. ASR102; Meridian Bioscience) and was assembled into a final PCR mixture as follows: 100 nM each TaqMan probe targeting *H. pylori* 23S rRNA in the green (emission wavelength, 510 nm) and red (emission wavelength, 660 nm) channels, 400 nM ASR primers, 40 nM control primers (catalog no. BIO-11025 [includes primers and probe]; Bioline), 100 nM control probe (emission wavelength, 610 nm), and 1× SensiPLUS master mix (catalog no. BIO-11021; Bioline). Typically, 20 μl of the purified DNA sample was added to the amplification mixture. Molecular-grade water was added to make up the total volume of the PCR mixture to 50 μl. PCR amplification was performed with the following parameters: an initial hold temperature of 95°C for 10 min and then 50 cycles of 95°C for 20 s and 65°C for 60 s, followed by a melting protocol measuring the fluorescent intensity of the red probe (emission wavelength, 660 nm) in the temperature range of 52°C to 70°C (10-s wait between intervals; 1°C increase with each cycle).

Analysis of PCR. PCR results for *H. pylori* detection were calculated based on the CRM as the comparator method. CLA resistance due to the presence of 23S rRNA mutations was predicted in parallel with the detection of *H. pylori*. Amplification observed in the green channel translated to the presence

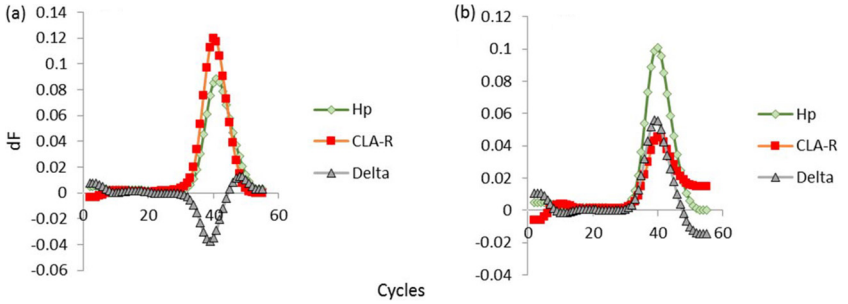

FIG 1 Fluorescent signal analysis for a wild-type (a) and a mutant (b) genotype.

of *H. pylori* in the purified DNA from stool. Amplification observed in the red channel was used for predicting CLA resistance or susceptibility. Two alternative approaches were employed to analyze amplified DNA and make a determination of CLA susceptibility or resistance for each sample. The first approach consisted of using differential fluorescent signal analysis and finding the derivate function (rate at fluorescent change) for both the green and the red signal for every cycle. The difference between the green and red signals determined the resistance prediction. If the red signal had an attenuated result and, therefore, a positive delta relative to the green signal, the sample was called as containing a mutation conferring clarithromycin resistance. If the red signal was the same as or (in most cases) stronger than the signal in the green channel, the result would be a negative delta relative to the green signal. The sample was then considered not to contain targeted mutations and to be susceptible to clarithromycin. Figure 1 shows examples for a sample that was predicted to be resistant to clarithromycin and a sample predicted to be susceptible.

The second approach used to analyze the *H. pylori* amplified target and to make a prediction with regard to resistance was melting curve analysis in the temperature range of 52°C to 72°C. A 10-s wait was programmed between intervals, and the temperature increased 1°C with each cycle. When a single peak was observed at temperatures lower than 60°C, typically 54 to 56°C, the sample was called as containing mutations conferring resistance to clarithromycin. When a single peak was observed above 60°C, typically 63 to 65°C, the sample was called as lacking targeted mutations and susceptible to clarithromycin. Figure 2 shows examples of typical melt profiles produced by the analysis of samples comprising wild-type sequences or different clarithromycin resistance mutations. Samples demonstrating amplification in the green channel and no amplification in the red channel were called as *H. pylori* positive and indeterminate for the presence of clarithromycin resistance mutations.

Purification of PCR products and sequencing. PCR products were purified using Macherey-Nagel's NucleoSpin gel purification kit (catalog no. 740609). The identity of amplified PCR products was confirmed by Sanger sequencing. GeneWiz (NJ, USA) performed bidirectional sequencing to confirm the genotype.

Reference testing. Patients were tested, in parallel to molecular testing, by the UBT and were subjected to esophagogastroduodenoscopy (EGD) followed by histology, culture, and a rapid urease test (RUT), producing a consensus patient infection status. PCR data were compared to the outcome of the eradication treatment for a subset of patients treated with clarithromycin as part of a combination regimen. All patients underwent a standard UBT at the beginning of antibiotic therapy and 4 to 6 weeks following the end of therapy. The UBT was performed after an overnight fast (14). This study was approved by the local ethics committee, informed consent was obtained from all the patients, and the

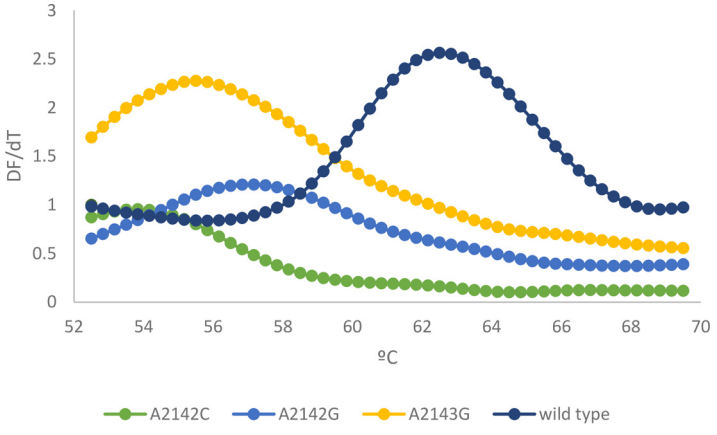

FIG 2 Melt curve analysis for the wild-type genotype and for the A2142G, A2142C, and A2143G mutant genotypes, which are associated with resistance to clarithromycin.

study was performed according to guidelines for good clinical practice and the Declaration of Helsinki (2013).

Statistical analysis. Means and their 95% confidence intervals were calculated. Comparisons among patient subgroups were performed using the chi-square test. Test accuracy was calculated by the "2-by-2" table method (MEDCALC, version 17.2). A P level of <0.05 was considered significant.

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